IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appln. Serial No.: ~~09/857,131~~ 10/049,783 Group Art Unit: 1654

Filing Date: 08/03/2001 Examiner: Roy Teller

Applicants: Fraser et al Attorney Docket No.: 78104.027

Title: Calcitonin for the Modulation of Sperm Function

DECLARATION OF LYNN R. FRASER UNDER RULE 132

Honourable Commissioner of Patents and Trademarks
Washington, DC 20231

Sir:

I, Lynn Repsis Fraser, do hereby declare as follows :

1. I am Professor of Reproductive Biology at the School of Biomedical Sciences, Kings College, University of London.

2. I have co-authored many published scientific papers in the field of research and technology to which the present application relates, including the following:

Green CM, Cockle SM, Watson PF and Fraser LR (1994) Stimulating effect of pyroglutamylglutamylprolineamide, a prostatic TRH-related tripeptide, on mouse sperm capacitation and fertilizing ability *in vitro*. *Mol Reprod Develop* **38**, 215-221.

Green CM., Cockle SM, Watson PF and Fraser LR (1996a) Fertilization promoting peptide, a tripeptide similar to thyrotrophin-stimulating hormone, stimulates the capacitation and fertilizing ability of human spermatozoa *in vitro* *Hum Reprod* **11**, 830-836.

Green CM, Cockle SM, Watson PF and Fraser LR (1996b) A possible mechanism of action for fertilization promoting peptide, a TRH-related tripeptide that promotes capacitation and fertilizing ability in mammalian spermatozoa. *Mol Reprod Dev* **45**, 244-252.

Fraser LR, Hanyaloglu A and Cockle SM (1997) A fertilization promoting peptide (FPP)-related tripeptide competitively inhibits responses to FPP - a cause of male subfertility? *Mol Reprod Dev* **48**, 529-535.

Fraser LR and Adeoya-Osiguwa SA (1999) Modulation of adenylyl cyclase by FPP and adenosine involves stimulatory and inhibitory a .osine receptors and G proteins. *Mol Reprod L* 53, 459-471.

Fraser LR, Pondel MC and Vinson GP (2001) Calcitonin, angiotensin II and FPP significantly modulate mouse sperm function. *Mol Hum Reprod* 7, 245-253.

Adeoya-Osiguwa SA and Fraser LR (2003) Calcitonin acts as a first messenger to regulate adenylyl cyclase/cAMP and mammalian sperm function. *Mol Reprod Dev* 65, 228-236.

3. I am a co-inventor of the subject matter described and claimed in the above-identified patent application. As such, I am intimately familiar with the contents of the application.

4. I have been made aware of the objections to the present application raised by the Examiner in the Office Action dated 05/19/2003 and my reply is as follows:

5. The Examiner has cited the Gnessi paper, to which reference has been made in the present application. The study published by Gnessi et al (1984), cited by the Examiner, is a poor one. Sperm suspensions were prepared in phosphate-buffered saline, a medium that does not support sperm capacitation and fertilization. Suspensions were mixed with either salmon or human calcitonin at various concentrations ranging from 4 x 10^{-9} M (4 nM) to 4 x 10^{-5} M (40 μM), incubated for 2 min and then evaluated for motility. The authors detected a decrease in sperm motility as the concentration of salmon, but not human, calcitonin increased.

6. In contrast, we prepared sperm suspensions in modified Tyrode's medium (for example page 2, line 6 of the application as filed) and we have found that both salmon (1.5 x 10^{-9} M = 1.5 nM) and human calcitonin (60 x 10^{-9} M = 60 nM) stimulated mouse sperm capacitation, as demonstrated by chlortetracycline (CTC) analysis, and sperm fertilizing ability, as demonstrated by in vitro fertilization. Our results have been published in the scientific literature (see Fraser et al, 2001, Reference list below). Our findings showed no obvious diminution of sperm motility and the functional responses we obtained would not occur if sperm motility had been compromised by treatment: successful fertilization requires that the sperm (1) be highly motile and (2) express 'hyperactivated' motility (required for penetration of the non-cellular zona pellucida that surrounds the unfertilized egg).

7. The calcitonins used in the study by Gnessi et al were a gift from another individual, not purchased from a recognized supplier of reagents such as Sigma-Aldrich, the source of the calcitonins we use; perhaps the salmon calcitonin had some contaminant.

8. More recently, we have investigated the responses of human sperm to salmon calcitonin. Semen samples were obtained from donors with normal semen parameters (good sperm concentration, motility and morphology). Sperm were prepared by centrifuging semen for 5 min at 600 g through a discontinuous gradient of Percoll (bottom layer, 95%; middle layer, 70%; top layer, 50% Percoll); the most motile sperm are found in the pellet at the bottom of the tube after centrifugation. The pelleted cells were collected and washed once by resuspending in Earle's medium containing 4 mg/ml human serum albumin and

centrifuging as previously; again pelleted cells were resuspended in fresh medium. The sperm concentration was assessed, adj d to 5 x 10^6 cells/ml and the suspension was t divided into several aliquots. To these were added: no peptide (untreated control), 100 nM fertilization promoting peptide (FPP; served as the positive control), and then salmon calcitonin at concentrations from 0.015 – 15 nM (0.05 – 50 ng/ml). Suspensions were incubated at 37° C in an atmosphere of 5% CO_2, 5% O_2, 90% N_2 for 1 h. A drop of each treated/control suspension was examined for motility; all samples had good motility and there was no evidence that the calcitonin-treated suspensions had reduced motility. Cells were then stained for chlortetracycline (CTC) assessment, fixed and slides were prepared (methods used were those described in Green et al, 1996, Reference list below); 5 replicate experiments were carried out.

9. CTC analysis revealed that salmon calcitonin, like FPP, significantly stimulated (**P<0.025, ***P<0.01) capacitation as evidenced by more B pattern (capacitated, potentially fertilizing) and fewer F pattern cells (uncapacitated, non-fertilizing). Concentrations from 1.5-15 nM (5-50 ng/ml) were as effective as 100 nM FPP. The data are shown in Figure 1, attached hereto. These results suggested that calcitonin-treated human sperm would be more fertile in vitro; it is not possible to obtain fresh, unfertilized human eggs for research purposes, so this was evaluated by testing the ability of calcitonin-treated sperm to penetrate zona pellucida-free hamster eggs (see Green et al, 1996, op cit); 5 replicate experiments were carried out. Both calcitonin- and FPP-treated human sperm were significantly (P<0.025) more fertile than untreated control sperm, with ~62% of oocytes penetrated by peptide-treated suspensions, compared with ~31% for the untreated control suspensions. If calcitonin had inhibited sperm motility, then we would not have obtained successful penetration since sperm need to be vigorously motile in order to succeed. Thus we have biologically important evidence that human sperm respond positively to salmon calcitonin used at concentrations within the range reported by Gnessi et al to have inhibited human sperm motility. These results confirm the validity of the teaching contained in our above-identified application as filed.

10. The Examiner objects that the application provides no working Example of the use of porcine calcitonin. However, use of porcine calcitonin is referred to in the last sentence of page 9 of the application as filed. There are three main classes of calcitonin: teleost/avian (includes salmon), artiodactyl (includes pigs) and rat/human (Pozvek et al, 1997w) (see page 1, paragraph 4 of the application as filed). The most potent class, in terms of biological activity on cells involved in bone homeostasis, is teleost/avian, the least potent is rat/human, and artiodactyl calcitonins are intermediate.

11. When designing experiments to investigate the effects of salmon and human calcitonin on mouse sperm, we postulated that the relative potencies would be similar to those observed with bone cells since calcitonin works by binding to a specific receptor and there is no evidence that sperm calcitonin receptors and bone cell calcitonin receptors are different. Therefore, we used 5 ng/ml salmon calcitonin and 200 ng/ml human calcitonin and obtained similar stimulatory responses (page 2, lines 9 to 10 of the application as filed); we also tried 20 ng/ml human calcitonin and observed less stimulation than with 200 ng/ml (Fraser et al, 2001, a copy of which is attached as Exhibit 1). Thus the relative biological activity of salmon and human calcitonins on sperm correlates with their known potencies on bone cells. Since the biological activity of

porcine calcitonin is intermediate between that of salmon and human calcitonins, a skilled person would be able, without any difficulty, to e. .polate the necessary information from the disc. ure in the application as filed, to determine the appropriate concentrations of porcine calcitonin to produce comparable effects on sperm function. Thus, on the basis of our results with salmon and human calcitonins, the concentration required would be higher than that for salmon and lower than that for human calcitonin. Hence, in the last sentence of page 9 of the application as filed, we recommend a final concentration of 200-500 ng/ml of porcine calcitonin for addition to semen samples as an alternative to 50-200 ng/ml of salmon calcitonin.

12. I declare that all statements herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the application or any patent issuing thereon.

REFERENCES

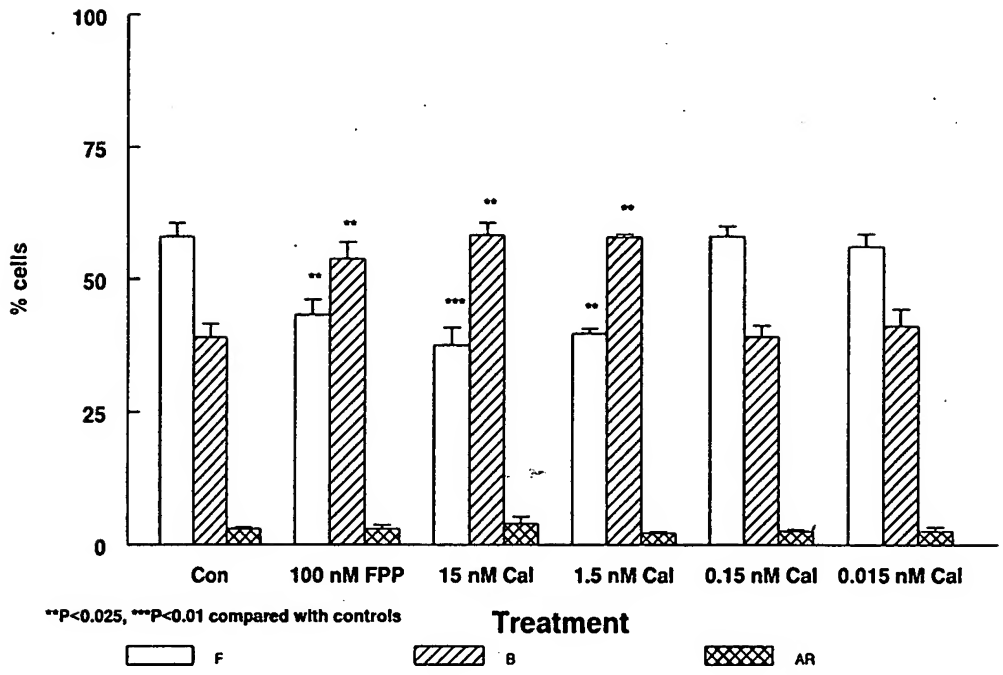
Gnessi L, Silvestroni L, Baffri A et al (1984) Salmon calcitonin inhibits human sperm motility in vitro. Biochem Biophys Res Commun 125, 199-204.

Green CM, Cockle SM, Watson PF and Fraser LR (1996) Fertilization promoting peptide, a tripeptide similar to thyrotrophin-stimulating hormone, stimulates the capacitation and fertilizing ability of human spermatozoa in vitro. Hum Reprod 11, 830-836.

Fraser LR, Pondel MC and Vinson GP (2001) Calcitonin, angiotensin II and FPP significantly modulate mouse sperm function. Mol Hum Reprod 7, 245-253.

Pozvek G, Hilton JM, Quiza M et al (1997) Structure/function relationships of calcitonin analogues as agonists, antagonists, or inverse agonists in a constitutively activated receptor cell system. Mol Pharmacol 51, 658-665.

Fig. 1. Human sperm responses to salmon calcitonin



Lynn R Fraser

7 August 2003

Date